CCLVI. THE OXIDATION OF *l*(−)α-HYDROXYGLUTARIC ACID IN ANIMAL TISSUES

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l(−)α-HYDROXYGLUTARIC ACID was first recognized as a substrate of the intermediary metabolism of animal tissues by Thunberg [1920] who found that it reduced methylene blue in presence of washed frog muscle. Wishart [1923], working with the methylene blue technique, obtained extracts from skeletal muscle of ox and rabbit which showed a very weak activity towards hydroxyglutaric acid, whereas Holmberg [1934] reported negative results with similar extracts. But apart from these few scattered observations the dehydrogenation of hydroxyglutaric acid seems to have passed unnoticed.

In a forthcoming paper evidence will be presented that, anaerobically, a dismutation takes place between 2 mol. of α-ketoglutaric acid, one of which is oxidized to succinic acid and CO_2 and the other reduced to *l*(−)α-hydroxyglutaric acid. This fact seemed to shed new light on the significance of *l*(−)α-hydroxyglutaric acid as a metabolite. A systematic study of its oxidation in animal tissues and of the properties of the oxidizing enzyme was therefore thought desirable.

I. *The oxidation of* l(−)α-*hydroxyglutaric acid by rat tissues*

The respiration of some tissues of the rat, in presence and in absence of *l*(−)α-hydroxyglutaric acid, is shown in Table I. The respiration of most tissues

Table I. *Respiration of rat tissues in presence and in absence of* l(−)α-*hydroxyglutaric acid. Phosphate saline. Slices, except diaphragm, intestine and testis*

Tissue	Hydroxyglutarate	Q_{O_2} 1st hr.	2nd hr.	3rd hr.
Brain	0	− 6·8	− 3·9	−2·1
	M/50	− 8·3	− 4·6	−3·4
Diaphragm	0	− 5·3	− 4·8	−4·0
	M/50	− 6·6	− 5·6	−4·7
Heart	0	− 7·0	− 6·4	−5·4
	M/50	−12·1	−10·4	−8·8
Intestine	0	− 4·2	− 2·8	−2·3
	M/50	− 4·0	− 2·3	−2·0
Jensen sarcoma	0	−10·6	− 9·3	−8·0
	M/50	−10·3	− 8·7	−7·9
Liver	0	− 9·8	− 9·2	—
	M/50	−10·9	−10·9	—
Kidney	0	−15·2	−11·5	—
	M/50	−24·0	−24·0	—
Spleen	0	−12·3	−10·4	−7·8
	M/50	−11·0	− 9·1	−7·5
Testis	0	− 5·1	− 3·6	—
	M/50	− 6·0	− 5·0	—

(especially heart and kidney) is substantially increased in presence of the substrate. No increase was found with spleen, intestine and Jensen sarcoma.

Measurement of the metabolism of rat kidney slices by the Dickens-Šimer bicarbonate technique [1931] revealed a rise of R.Q. from 0·84 to about unity and a considerable disappearance of acid groups in presence of hydroxyglutaric acid, facts which indicate a combustion of the substrate to CO_2 and H_2O (Table II). If the metabolism of hydroxyglutaric and of α-ketoglutaric acid is compared with the metabolism of lactic and pyruvic acids in slices of rat kidney [Elliott *et al.* 1935], a close analogy appears of the figures for the two hydroxy-acids and of those for the two keto-acids. As has been pointed out by Elliott *et al.*, it is difficult to account for the disappearance of acid groups merely on the basis of an oxidative removal.

Table II. *Metabolism of rat kidney slices in presence and in absence of* (a) l(−)α-*hydroxyglutaric and* (b) α-*ketoglutaric acid. Dickens-Šimer bicarbonate method. Duration of exp.: 3 hr.*

Substrate	Q_{O_2}	Q_{CO_2}	R.Q.	Q_{Ac}*	Theoret. R.Q.
0	−17·0	14·2	0·84	− 1·50	—
M/50 hydroxyglutarate	−28·6	28·0	0·98	− 9·55	1·11
0	−18·5	15·6	0·84	− 1·50	—
M/50 α-ketoglutarate	−38·5	46·4	1·20	−21·5	1·25

* $Q_{Ac} = Q_{Acid\ groups}$.

d(+)α-Hydroxyglutaric acid does not increase the respiration of tissue slices (Table III).

Table III. *Respiration of rat tissue slices in presence and in absence of* d(+)α-*hydroxyglutaric acid. Phosphate saline*

Tissue	Substrate	Q_{O_2} 1st hr.	2nd hr.	3rd hr.
Brain	0	− 6·7	− 2·9	− 1·3
	M/50 *d*(+)α-hydroxyglutaric acid	− 6·7	− 2·8	− 1·5
Kidney	0	−18·3	−16·6	−15·2
	M/50 *d*(+)α-hydroxyglutaric acid	−17·8	−16·2	−15·5

II. *The properties of hydroxyglutaric dehydrogenase*

(1) *Preparation of the enzyme.* Pig's heart is the most suitable source of active extracts. The muscular tissue of one heart is minced, the mince thoroughly washed with tap water and finely ground with sand. The resulting paste is extracted for 20 min. at 37° in slightly alkaline medium. Most of the extracts used for the experiments to be described have been prepared with 5% $Na_2HPO_4,12H_2O$, though it was found later that phosphate extraction is somewhat injurious to the enzyme. The most active preparations have been obtained by extracting the pulp from one heart with about 200 ml. *M*/10 $NaHCO_3$ (Table IV). After squeezing through linen the extract is brought to *p*H 4·6 by addition of *M*/10 acetic acid (approx. 80 ml.). The precipitate is centrifuged off and washed twice with distilled water. It is finally resuspended in about 20 ml. *M*/15 veronal buffer of *p*H 8·2. In this form the enzyme is stable for about a week if kept at 0°. The precipitate can be dried, and the dry enzyme, if kept *in vacuo* at 0°, is stable for a much longer period. If the drying is carried

out quickly, there is little loss of activity. The precipitate is spread in a thin layer on Petri dishes and dried over P_2O_5 in an efficient vacuum, the P_2O_5 being renewed after a few hours.

Table IV. *Comparison of the activities of different extracts*

40 g. of heart pulp extracted with 100 ml. of solution, extract adjusted to *p*H 4·6 and precipitate resuspended in *M*/15 veronal buffer *p*H 8·2. Final volume of suspension 20 ml. in each case. 2 ml. enzyme solution, 0·3 ml. *M*/50 pyocyanine and 0·2 ml. *M* *l*(−)α-hydroxyglutarate per vessel.

Extracted with 100 ml. of	μl. O_2 uptake in 1 hr.
M/10 bicarbonate	262
M/40 bicarbonate + 5% CO_2 (*p*H 7·4)	209
M/35 veronal buffer *p*H 8·0	202
5% $Na_2HPO_4,12H_2O$	147
M/10 phosphate buffer *p*H 7·4	135

(2) *The reaction with oxygen.* The mixture of enzyme and substrate takes up O_2 vigorously if pyocyanine is present as carrier. In presence of sufficient substrate the O_2 uptake does not fall off markedly for several hours. The system requires neither coenzymes nor a ketone fixative.

(3) *The product of oxidation.* (*a*) End value of O_2 uptake: the O_2 uptake stops when exactly 0·5 mol. O_2 has been absorbed for 1 mol. of substrate (Fig. 1).

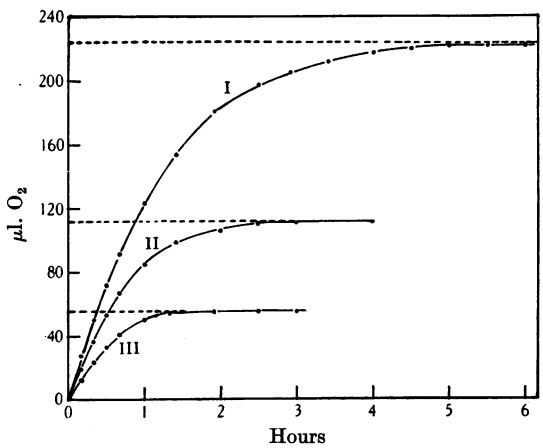

Fig. 1. End value of O_2 uptake. 2 ml. enzyme, 0·5 ml. *M*/50 pyocyanine. I, 0·2 ml. *M*/10 *l*(−)α-hydroxyglutarate; II, 0·1 ml. *M*/10 *l*(−)α-hydroxyglutarate; III, 0·1 ml. *M*/20 *l*(−)α-hydroxyglutarate. The dotted lines indicate the level of the theoretical end value for 0·5 mol. O_2 per mol. of hydroxyglutarate.

(*b*) Formation of carbonyl groups: it can be shown that for each mol. of O_2 absorbed 2 CO— equiv. appear. CO— groups were estimated in the trichloroacetic acid filtrate by titration of the bisulphite-binding power, using the modified method of Elliott *et al.* [1935]. Instead of solid Na_2HPO_4 which dissolves very slowly, 10 ml. of *M*/2 solution have been used. Pyocyanine does not combine with bisulphite under the conditions employed, and its faint blue colour does not interfere with the iodine titration if a darker blue is chosen as the end point (Table V).

Table V. *Formation of carbonyl groups during hydroxyglutarate oxidation*

2 ml. enzyme, 0·3 ml. *M*/50 pyocyanine and 0·3 ml. *M*/2 hydroxyglutarate per vessel.

Time of incubation hr.	μl. O_2 uptake (corrected for blank)	μl. carbonyl	Ratio O_2 : carbonyl
0	—	0	—
1	130·5	252	1 : 1·93
2	242	484	1 : 2·0
3	339	668	1 : 1·97
4	434	880	1 : 2·03

(*c*) Isolation of α-ketoglutaric acid: the absorption of 0·5 mol. O_2 and the formation of 1 CO— equiv. per mol. of hydroxyglutaric acid are consistent with the supposition that α-hydroxyglutaric acid is oxidized to α-ketoglutaric acid. Final proof was obtained by the isolation of the latter as 2:4-dinitrophenylhydrazone.

To the extract from two hearts (120 ml.) were added 20 ml. *M*/50 pyocyanine and 20 ml. *M* hydroxyglutarate. The mixture was incubated at 37·5° for 7 hr. and O_2 was bubbled through at frequent intervals. After deproteinizing with trichloroacetic acid, 100 ml. of 1 % 2:4-dinitrophenylhydrazine in 2*N* HCl were added to the filtrate. After 2 hr. at 0° the yellow precipitate was filtered off, redissolved in 8 % $NaHCO_3$ and the filtrate again precipitated with HCl. The product (1·164 g.) was twice recrystallized from ethyl acetate-ligroin mixture and 0·950 g. of pure α-ketoglutaric-2:4-dinitrophenylhydrazone was obtained. M.P. 223°, mixed M.P. 223° (uncorr.). (Found (Weiler): C, 40·69; H, 3·16; N, 17·2 %. Calc. for $C_{11}H_{10}O_8N_4$: C, 40·44; H, 3·09; N, 17·18 %.)

(4) *Kinetics.* (*a*) Type of reaction: the substrate concentration, calculated from the absorption of O_2 and expressed logarithmically, decreases linearly, indicating a unimolecular reaction.

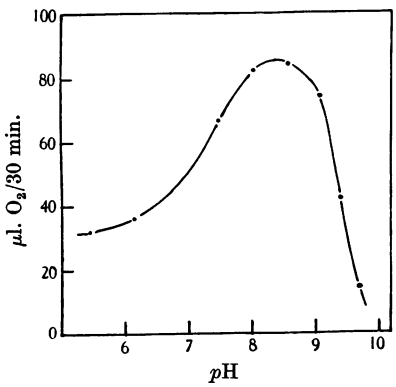

Fig. 2.

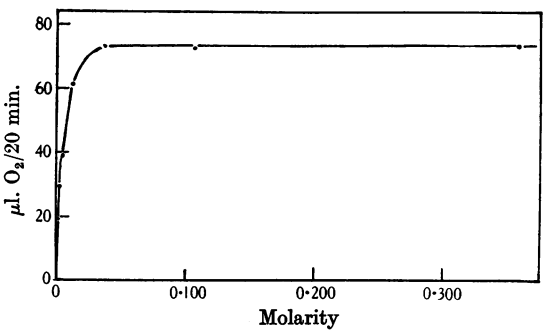

Fig. 3.

Fig. 2. *p*H optimum. 1 ml. enzyme, suspended in water and neutralized with NaOH. 1 ml. *M*/10 buffer (veronal buffer up to *p*H 8·5, glycine buffer for *p*H > 8·5), 0·3 ml. *M*/50 pyocyanine, 0·2 ml. *M* hydroxyglutarate.

Fig. 3. Rate of reaction with varying substrate concentration. 2 ml. enzyme, 0·3 ml. *M*/50 pyocyanine. Solutions of hydroxyglutarate and water to make total volume of 3 ml.

(*b*) *p*H optimum: Fig. 2 shows the dependence of the rate of reaction on the *p*H of the solution. The optimum velocity is observed at *p*H 8·0–8·5, falling off rapidly in the more alkaline range.

(*c*) Influence of substrate concentration: the substrate concentration at which half the maximum velocity is attained is about *M*/300. Full saturation of the enzyme is reached with a substrate concentration of *M*/30. Higher substrate concentrations do not inhibit (Fig. 3).

(5) *Optical specificity.* *d*(+)α-Hydroxyglutaric acid is not attacked by the enzyme. Our sample of *d*(+)α-hydroxyglutaric acid was only 92% optically pure ($[\alpha]_D$ of Na salt 7·2° instead of 8·6°). The small oxygen uptake is entirely accounted for by the contamination with *l*(−)α-hydroxyglutaric acid (Table VI).

Table VI. *Activity of the enzyme towards the optical isomerides*

2 ml. enzyme, 0·3 ml. *M*/50 pyocyanine, 0·3 ml. *M*/5 substrate per vessel.

Substrate	μl. O_2 uptake 2 hr.
l(−)α-Hydroxyglutarate	472
d(+)α-Hydroxyglutarate	52

(6) *Formation of H_2O_2.* The rate of reaction is apparently increased in the presence of cyanide. The effect varies with the concentration of cyanide, the concentration of the enzyme and the origin of the extract. It is most marked with extracts from brain, where the initial velocity can be more than doubled. But whereas the O_2 absorption is fairly linear in absence of cyanide, there is a marked fall in its presence (Fig. 4). The effect of cyanide is less with extracts from kidney or heart. Dilution of the enzyme increases it (Table VII). The optimum concentration of cyanide is about *M*/30 (Table VIII). With lower concentrations a short initial acceleration of the O_2 uptake is seen which is soon replaced by the normal rate.

Table VII. *Influence of enzyme concentration on cyanide effect*

Extract from pig's heart. 0·2 ml. *M*/2 hydroxyglutarate and 0·3 ml. *M*/50 pyocyanine per vessel. Water to make 3 ml.

	μl. O_2 uptake (1 hr.)		
ml. enzyme	Without 0·2 ml. *M* cyanide	With 0·2 ml. *M* cyanide	% increase
0·5	36	53	47
2·0	218	266	22

Table VIII. *Influence of cyanide concentration on the effect of cyanide*

Extract from pig's heart. 1 ml. enzyme, 0·5 ml. *M*/50 pyocyanine, 0·3 ml. *M* Hydroxyglutarate per vessel. Water to make 3·2 ml.

Concentration of cyanide	μl. O_2 uptake 1 hr.	% increase	$Ti(SO_4)_2$ reaction
M/7	246	20·5	+ + +
M/30	258	26·5	+ +
M/150	240	17·5	+
M/450	214	5	0
0	204	—	0

The theoretical end value of the O_2 uptake is considerably exceeded in presence of cyanide, though the reaction proceeds very slowly after the first 2 hr. and the O_2 uptake does not reach 200% of the theoretical value (Fig. 5).

These facts suggest that the accelerating effect of cyanide is due to inhibition of catalase and accumulation of H_2O_2. The presence of H_2O_2 was repeatedly demonstrated by the colour reaction with titanium sulphate: after depro-

teinization with trichloroacetic acid the filtrate was made alkaline and pyocyanine was extracted exhaustively with chloroform. The colourless solution turned yellow on acidification and addition of a few drops of titanium sulphate solution. The controls without cyanide remained colourless.

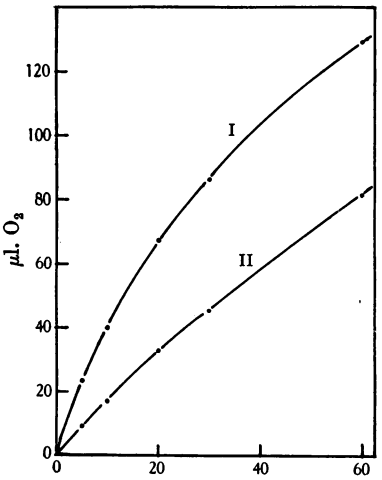

Fig. 4.

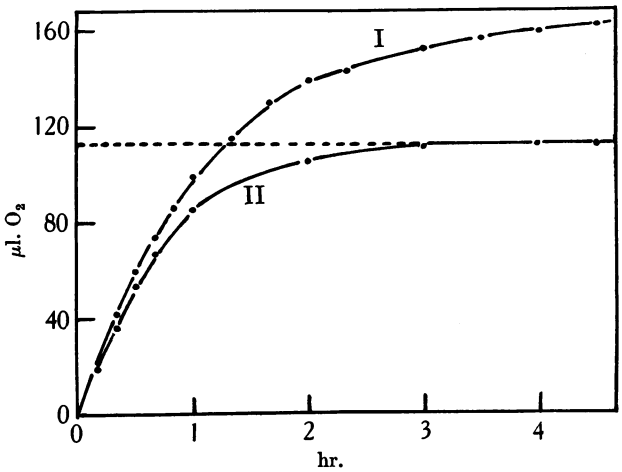

Fig. 5.

Fig. 4. Effect of cyanide on O_2 uptake. 2 ml. enzyme from horse brain, 0·3 ml. *M*/50 pyocyanine, 0·3 ml. *M* hydroxyglutarate. I with, II without 0·1 ml. *M* cyanide.

Fig. 5. End value of O_2 uptake in presence of cyanide. 2 ml. enzyme from pig's heart, 0·5 ml. *M*/50 pyocyanine, 0·1 ml. *M*/10 hydroxyglutarate. I with, II without 0·5 ml. *M* cyanide. The dotted line indicates the level of the theoretical end value.

The formation of H_2O_2 in reactions where an autoxidizable carrier is concerned is not surprising [cf. Reid, 1930]. It has been demonstrated for the hexosemonophosphate system with "yellow enzyme" as carrier [Warburg & Christian, 1933] and recently for the glycerophosphoric dehydrogenase with pyocyanine, methylene blue or brilliant cresyl blue as carrier [Weil-Malherbe, 1937, 2].

The dehydrogenases which react directly with molecular O_2 without the intermediation of a carrier ("aerobic" dehydrogenases) also form H_2O_2. As Keilin & Hartree [1936] have shown, the H_2O_2 can in these cases be utilized in secondary "coupled" oxidations, if catalase and a suitable substrate, e.g. the Nadi reagent or alcohol or haemoglobin [Bernheim *et al.* 1936], is present. It is often held that this coupled oxidation is an indicator for the formation of H_2O_2. But although H_2O_2 is formed in the reactions quoted above, no coupling occurs in these cases. In the hydroxyglutaric system an O_2 uptake exceeding the theoretical value was never observed in absence of cyanide and could never be provoked by addition of alcohol. The mechanism which brings about the coupling, when "aerobic" dehydrogenases form H_2O_2, seems to be lacking in the "anaerobic" dehydrogenases.

(7) *Carriers.* A large number of reversible redox systems, artificial and natural, have been tested for their carrier properties in the hydroxyglutaric acid-hydroxyglutaric dehydrogenase system. Of those tested pyocyanine and a few closely related substances were by far the most efficient carriers (Table IX).

This extraordinary specificity of the phenazine compounds is perhaps the strangest property of this enzyme. Dickens (unpublished) has found a similar superiority of phenazine compounds, compared with other artificial carriers, in the oxidation of hexosemonophosphate and phosphohexonate by yeast enzymes, though here "yellow enzyme" is an even more efficient carrier. In other dehydrogenase systems pyocyanine is not superior to other carriers, e.g. in the lactic [Green & Brosteaux, 1936] or malic dehydrogenase systems [Green, 1936], and even inhibits succinic dehydrogenase [Weil-Malherbe, 1937, 1].

Table IX. *Carrier activity of artificial oxidation-reduction indicators*

2 ml. enzyme, 0·3 ml. *M*/2 hydroxyglutarate, 0·5 ml. *M*/100 redox indicator per vessel. All values corrected for blanks.

Carrier	E_0' at *p*H 7·0	μl. O_2 uptake 1 hr.
0	—	22
Pyocyanine	−0·034	202
Thionine	+0·062	49
Brilliant cresyl blue	+0·045	39
Methylene blue	+0·011	33·5
Gallocyanine	+0·021	26
Methyl Capri blue	−0·061	21
Lactoflavin	−0·215	15
Phenosafranine	−0·256	14
Prune	+0·05	14
Acridine methochloride	?	9·5

The potentials of many of the tested indicators are more positive than or very close to that of pyocyanine. They should therefore be thermodynamically capable of replacing pyocyanine. Its superiority must be attributed to its chemical properties.

Phenazine methochloride shows an activity equal to that of pyocyanine; the corresponding ethyl compounds were found to be even slightly more active (Table X). The *N*-methyl- and *N*-ethyl-phenazines are autoxidizable at *p*H > 7, the oxidation product being inactive. Experiments with these compounds have therefore to be carried out at *p*H 7. Even then a slow autoxidation takes place which was corrected for.

Table X. *Comparison of the carrier activities of* N-*methyl- and* N-*ethyl-phenazine and* N-*methyl- and* N-*ethyl-oxyphenazine*

2 ml. enzyme, 0·3 ml. *M*/2 hydroxyglutarate and 0·5 ml. of 0·25% phenazine compound per vessel. *p*H = 7·0. Values corrected for blanks.

Carrier	μl. O_2 uptake 40 min.
N-Methyl-α-oxyphenazine (pyocyanine)	115
N-Ethyl-α-oxyphenazine	135·5
Phenazine methochloride	111·5
Phenazine ethosulphate	127

Examination of a series of other phenazine compounds did not reveal any carrier of comparable activity (Table XI). It is noteworthy that the shifting of the oxygen atom from the α- to the β-position results in a complete loss of activity.

In the systems studied by Dickens phenazine methochloride was greatly superior to pyocyanine. No such difference was found in the hydroxyglutaric system. The experiments of Table XII and Figs. 6 and 7 were made with different enzyme preparations and at different *p*H and are not strictly comparable, but

Table XI. *Carrier activity of phenazine compounds*

2 ml. enzyme, 0·3 ml. *M*/2 hydroxyglutarate and 0·5 ml. of 0·5% phenazine compound per vessel. *p*H = 8·2.

Carrier	μl. O_2 uptake 1 hr.
0	7
Pyocyanine (*N*-methyl-α-oxyphenazine)	196
N-methyl-β-hydroxyphenazine	12
N-ethyl-β-hydroxyphenazine	9
0	27
Pyocyanine	193
N-methyl-*NN'*-dihydrophenazine-2-sulphonic acid	47
2-Aminophenazinemethosulphate	46
Na *N*-methylphenazinedisulphonate	46
Chlororaphine	29

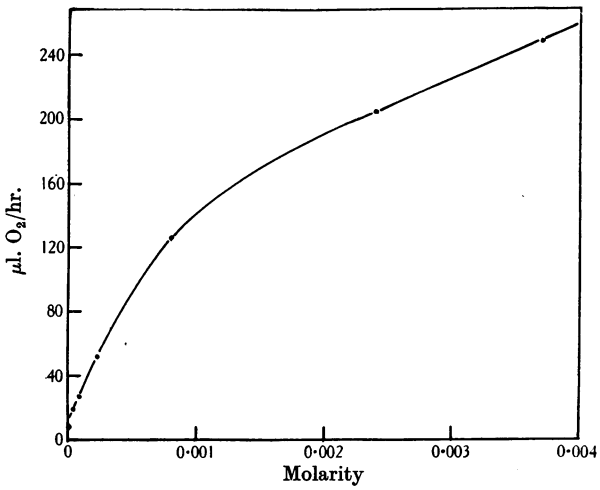

Fig. 6. Activity curve of pyocyanine. 2 ml. enzyme (*p*H 8·2), 0·2 ml. *M* hydroxyglutarate, solutions of pyocyanine and water to make a total volume of 3 ml.

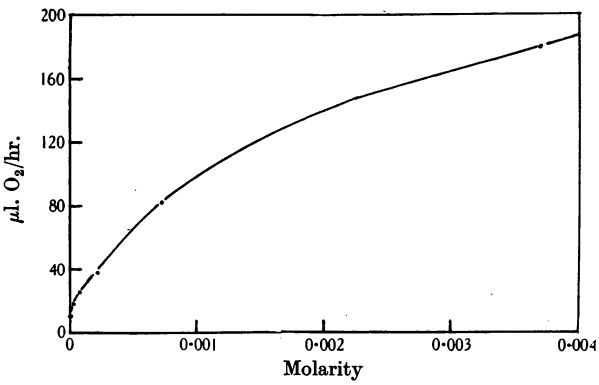

Fig. 7. Activity curve of phenazine methochloride. 2 ml. enzyme (*p*H 7·0), 0·2 ml. *M* hydroxyglutarate, solutions of phenazine methochloride and water to make a total volume of 3 ml.

it is clear enough that the two curves are of the same type. The activity increases fairly linearly with increasing concentration of either catalyst throughout the range of concentrations studied. The turnover numbers (mol. O_2 per 0·5 mol. catalyst per min.) are of the same order of magnitude.

Table XII. *Catalytic activities of varying concentrations of pyocyanine and phenazine methochloride*

2 ml. enzyme and 0·2 ml. *M* hydroxyglutarate per vessel. Carrier solution and water to make 2·7 ml. *p*H = 8·2 in the case of pyocyanine and 7·0 in the case of phenazine methochloride. Values corrected for blanks.

μl. pyocyanine 210 mg. = 22400 μl.	μl. O_2 uptake 1 hr.	Turnover no. average over 1st hr.
0	6	—
2·24	15·5	0·14
4·48	22	0·12
13·4	41·5	0·09
44·8	119	0·08
134	211	0·05
224	248	0·04
μl. phenazine methochloride 230·5 mg. = 22400 μl.		
0	10·8	—
2·24	17·1	0·09
4·48	21·9	0·08
13·4	37·5	0·07
44·8	82·3	0·05
224	178·7	0·03

None of the carriers mentioned above is known to be a normal constituent of animal cells. A number of natural redox systems have therefore been tested to ascertain if any of them can link the dehydrogenase with oxygen in the living cell. The results with cytochrome *c*, "yellow enzyme", adrenaline, ascorbic acid and glutathione (GSH) were completely negative. The same enzyme preparation was used as source of succinic and hydroxyglutaric dehydrogenases; whereas cytochrome *c* brought about a tenfold increase of succinate oxidation, it was without effect on the hydroxyglutarate oxidation (Table XIII). This shows that the inactivity of cytochrome *c* in the latter case was not due to saturation of the enzyme preparation with cytochrome *c*.

Table XIII. *The action of cytochrome* c *on the oxidation of hydroxyglutarate and succinate*

2 ml. of a 10% solution of dried enzyme in *M*/15 veronal buffer *p*H 8·2 per vessel.

Substrate	Catalyst	*M* cyanide	μl. O_2 uptake 1 hr.
0·3 ml. *M*/2 hydroxyglutarate	0	0	32
	0	0·2 ml.	35
	0·5 ml. cytochrome C	0	30
	0·5 ml. *M*/50 pyocyanine	0	232
			μl. O_2 uptake in 10 min.
0·3 ml. *M*/2 succinate	0	0	29
	0	0·2 ml.	0
	0·5 ml. cytochrome C	0	390

A slow oxidation of hydroxyglutarate by the enzyme takes place even if no carrier is added. This blank reaction varies between 5 and 30 μl. per hour. It is not influenced by cyanide, in strong contrast to the blank oxidation of succinic acid which is completely suppressed by cyanide (Table XIII). This affords additional evidence that cytochrome *c* does not react with this enzyme.

The problem arises, by what mechanism is the blank oxidation of hydroxyglutarate catalysed? It is very improbable that it is a direct reaction of the dehydrogenase with molecular O_2. It would be of great importance for the elucidation of the natural activation mechanism of this enzyme to identify the carrier concerned. Its insensitivity towards cyanide rules out indophenol oxidase as part of the carrier system. But cytochrome components other than *c* are perhaps autoxidizable under the conditions of the experiment. Spectroscopic analysis which was kindly carried out by Dr T. Mann, Cambridge, revealed indeed an almost instantaneous appearance of the band of reduced cytochrome *a* on addition of the substrate, which disappeared again on shaking with air. It is clear that the blank reaction is only a fraction of the maximum activity of the enzyme, and we have to assume that certain links in the carrier system have been removed or destroyed during the preparation. In spite of the inactivity of cytochrome *c*, the assumption is not improbable that, within the cell, the hydroxyglutaric dehydrogenase is linked with oxygen by a chain of cytochrome components.

(8) *Activators and inhibitors. Coenzymes.* The action of hydroxyglutaric dehydrogenase requires no coenzyme. Addition of coenzyme I (diphosphopyridinenucleotide), coenzyme II (triphosphopyridinenucleotide), adenosinetriphosphate or of a crude mixture of coenzymes from horse blood did not increase the activity. Table XIV shows the effect of adding coenzyme I on the oxidation of hydroxyglutarate, β-hydroxybutyrate and lactate by the same extract, demonstrating the absence of coenzyme from the enzyme preparation and the activity of the coenzyme preparation.

Table XIV. *Effect of coenzyme I on the oxidation of hydroxyglutarate, β-hydroxybutyrate and lactate*

2 ml. of the same extract per vessel. The hydroxyglutaric system contained in addition 0·2 ml. of *M* substrate, the β-hydroxybutyric system 0·2 ml. of *M* substrate (*dl*-) and 0·5 ml. of *M*/10 pyrophosphate, the lactic system 0·2 ml. *M* substrate, 0·2 ml. of *M* cyanide and 0·1 ml. of *M*/50 pyocyanine.

System	μl. O_2 uptake in 30 min. Without 0·5 ml. of 0·2% coenzyme I solution	With 0·5 ml. of 0·2% coenzyme I solution
Hydroxyglutaric	5	7·5
Hydroxyglutaric + 0·3 ml. *M*/50 pyocyanine	110	108
β-Hydroxybutyric	0	101·5
Lactic	2	249·5

Several other combinations were tried: e.g. yellow enzyme was used together with coenzyme I or II; ascorbic acid or glutathione or both together were added in presence of pyocyanine, but no activation could ever be observed. Furthermore, it could be shown that no diffusible or dissociable component is contained in extracts from unwashed tissue. When these were dialysed for different periods the rate of hydroxyglutarate oxidation did not diminish, but rather increased

(Table XV). Similarly the blank reaction rose, the more often the *p*H 4·6-precipitate was washed with distilled water (Table XVI). This shows that no soluble coenzyme or carrier has been removed.

Table XV. *Influence of dialysis on the oxidation of hydroxyglutarate by a phosphate extract of sheep's kidney*

50 g. of minced sheep's kidney incubated with 200 ml. of 5% $Na_2HPO_4,12H_2O$ at 37·5° for 20 min. Extract dialysed in cellophane sac against running tap water. 2 ml. extract per vessel.

	μl. O_2 uptake in 1 hr.	
Extract	Without 0·3 ml. *M*/2 hydroxyglutarate	With 0·3 ml. *M*/2 hydroxyglutarate
Not dialysed	48	56
3 hr. dialysis	31	69·5
16 hr. dialysis	23	66

Table XVI. *Influence of washing the enzyme precipitate* (p*H* 4·6) *on the blank reaction*

The precipitate of one extract was divided into 3 equal parts. After washing they were resuspended in equal volumes of *M*/15 veronal buffer *p*H 8·2. 2 ml. of enzyme solution per vessel.

	μl. O_2 uptake (2 hr.)	
Number of washings	Without 0·3 ml. *M*/2 hydroxyglutarate	With 0·3 ml. *M*/2 hydroxyglutarate
0	7·5	6
1	11·5	17
2	7	38

The actions of various inhibitors and of structurally related substances are summarized in Table XVII. The strongest inhibitors found were iodoacetic and oxalic acids. α-Ketoglutaric acid has but a small effect. The small inhibition by pyrophosphate is worth mentioning in view of the paper of Leloir & Dixon [1937], who found that only succinic dehydrogenase was inhibited by pyrophosphate.

Table XVII. *Action of inhibitors on the hydroxyglutaric system*

2 ml. enzyme, 0·3 ml. *M*/2 hydroxyglutarate and 0·3 ml. *M*/50 pyocyanine per vessel.

Inhibitor	% inhibition
M/60 arsenious oxide	6
M/35 sodium fluoride	0
M/60 iodoacetic acid	81·5
M/30 oxalic acid	83
M/20 tartronic acid	34
M/20 maleic acid	16
M/20 malonic acid	27
M/20 α-ketoglutaric acid	20
M/60 pyrophosphate	22
M/30 citric acid	20
M/20 *l*(+)-glutamic acid	0
M/30 *d*(+)α-hydroxyglutaric acid	0
M/30 glutaric acid	0

(9) *Distribution.* A comparison of extracts obtained from different sources was made in the following way: the tissue was minced, the mince washed and finely ground with sand in the usual way. The resulting paste was weighed

(g grams) and incubated with 2 parts of 5% $Na_2HPO_4,12H_2O$ at 37·5° for 20 min. The extract, clarified by centrifuging, was adjusted to pH 4·6 with acetic acid, the precipitate was centrifuged, washed and resuspended in $M/15$ veronal buffer of pH 8·2. The volume of the final suspension was measured (v ml.). 2 ml.+0·3 ml. $M/50$ pyocyanine were incubated with and without the substrate (0·3 ml. $M/2$ hydroxyglutarate) and the O_2 uptake measured for 1 hr. The difference of the two values (x μl.) divided by the ratio g/v is called activity quotient in Table XVIII (activity quotient $= xv/g$).

Table XVIII. *Comparison of the contents of hydroxyglutaric dehydrogenase in different tissue extracts*

Experimental arrangement, see text.

Animal	Tissue	Activity quotient
Pig	Heart	130
Rabbit	Heart	91
	Kidney	53
	Liver	30
	Brain	13·4
	Lung	3·6
	Skeletal muscle (white)	2·5
	Spleen	0
Rat	Heart	58
	Liver	30
	Spleen	0
	Jensen sarcoma	0

The enzyme was found in almost every organ with the exception of spleen and Jensen sarcoma. There is thus a fairly close agreement with the results of the tissue slice experiments.

(10) *Reversibility and potential.* The anaerobic reduction of a series of redox potential indicators by the hydroxyglutaric system has been tested in Warburg manometer vessels in an atmosphere of N_2 with a stick of yellow phosphorus in the centre well. Substrate+dye were added from the side bulb after the absorption of traces of O_2 had ceased. The following indicators with E_0' above −0·04 were completely reduced both by hydroxyglutaric acid alone and by mixtures of hydroxyglutaric and ketoglutaric acids up to a ratio of 1 : 9: thionine, brilliant cresyl blue, methylene blue and pyocyanine. Methyl Capri blue ($E_0' = -0{\cdot}061$) was completely reduced by hydroxyglutaric acid, but only partially by a 1 : 4 mixture of hydroxyglutaric and ketoglutaric acids. Ethyl Capri blue ($E_0' = -0{\cdot}072$) was only partially reduced by hydroxyglutaric acid and not at all by a mixture of hydroxy- and keto-glutaric acids. No reduction was observed with a series of indicators of a more negative potential. These results are such as would be expected for a thermodynamically reversible redox system with a normal potential in the region of −0·07. But it was never possible to demonstrate the reversibility directly by adding an excess of the oxidant after decoloration of the indicator by the reductant was completed. The indicators whose potential is closest to the expected potential of the enzyme system are methyl and ethyl Capri blue. They belong to those carriers which, if tested aerobically, even appear to inhibit the reaction. Their toxic action may cause the enzyme to lose its activity before reoxidation of the dye can occur. The direct electrometric measurement of the potential of the system was of course frustrated by the impossibility of finding a suitable indicator.[1]

[1] I wish to thank Dr D. E. Green for his helpful advice in this matter.

The reversibility of the system was, however, demonstrated by an indirect method. Dewan & Green [1937] and Green & Dewan [1937] found that coenzyme I has a very negative potential and that its reduced form can be oxidized by the oxidant of systems which, by virtue of their too positive potentials, are unable to reduce oxidized coenzyme I, e.g. the succinic system. They showed that coenzyme I links this and other systems with more negative donator systems, e.g. the β-hydroxybutyric system.

The hydroxyglutaric system can be linked in this way with the hydroxybutyric system. If α-ketoglutaric acid, β-hydroxybutyric acid and coenzyme I are incubated anaerobically with the enzyme mixture, some oxidation of hydroxybutyric to acetoacetic acid occurs. It must be concluded that a reduction of ketoglutaric to hydroxyglutaric acid corresponds to this oxidation. The effect is smaller than with pyruvic or fumaric acid, but it is outside any possible experimental error and gives sufficient proof of the reversibility of the hydroxyglutaric system (Table XIX). For these experiments, the precipitated enzyme was resuspended in a phosphate buffer *p*H 7·4, as the β-hydroxybutyric dehydrogenase is very susceptible to a more alkaline medium. The estimation of acetoacetic acid was carried out according to Dewan & Green [1937].

An attempt was made to link the hydroxyglutaric system by coenzyme I with the lactic system as donator. Provision had to be made for removal of any pyruvic acid formed from the reaction mixture to eliminate its inhibiting influence on the reaction [Green & Brosteaux, 1936]. Since ketone fixatives could not be used in the presence of α-ketoglutaric acid, a preparation of carboxylase made from Newcastle Brewery's top yeast according to Axmacher & Bergstermann [1934] was added. Pyruvic acid was decomposed by this preparation most vigorously whereas α-ketoglutaric acid was attacked much more slowly [cf. Westerkamp, 1933]. Any formation of pyruvic acid should therefore have led to an increased CO_2 evolution compared with that from α-ketoglutaric acid alone. The result, however, was negative.

Table XIX. *Oxidoreduction between the β-hydroxybutyric system and various acceptor systems*

	1	2	3	4	5	6
ml. enzyme suspension	2·0	2·0	2·0	2·0	2·0	2·0
ml. 0·3% coenzyme I	0·5	0·5	—	0·5	0·5	0·5
ml. *M dl*-β-hydroxybutyrate	0·2	—	0·2	0·2	0·2	0·2
ml. *M*/2 α-ketoglutarate	—	0·2	0·2	0·2	—	—
ml. *M*/2 pyruvate	—	—	—	—	0·2	—
ml. *M*/2 fumarate	—	—	—	—	—	0·2
ml. water	0·2	0·2	0·5	—	—	—
μl. acetoacetic acid (90 min.)						
Exp. 1	17	0	0	50	135	111
Exp. 2	13	0	0	78	236	92
Exp. 3	30	—	—	67	227	100

Discussion

Classification of the enzyme. Green & Brosteaux [1936] have divided the anaerobic dehydrogenases into two classes, the cytochrome- and the coenzyme-dehydrogenase systems. Hydroxyglutaric dehydrogenase seems to fit into neither of these categories. The evidence that neither coenzyme I nor coenzyme II catalyses the reaction is conclusive. But on the other hand it has to be borne in mind that the addition of cytochrome *c* is only a very fragmentary reconstruction of the full cytochrome catalysis in the living cell, where at least two

more components, cytochromes A and B, participate. It is therefore possible that hydroxyglutaric dehydrogenase belongs to the class of cytochrome dehydrogenases in spite of the impossibility of linking it with cytochrome C *in vitro*. The fact that cytochrome A is rapidly reduced by the system certainly supports this hypothesis.

The origin of α-hydroxyglutaric acid in the animal metabolism. The reduction of α-ketoglutaric acid is the only established reaction in animal metabolism which leads to the formation of *l*(−)α-hydroxyglutaric acid. An enzyme has been described in yeast [Neuberg & Collatz, 1930], in *B. coli* [Veibel, 1931] and in pea seedlings [Mayer, 1931], which brings about a dismutation of methylglyoxalylacetic acid to α-hydroxyglutaric acid. But it is the enantiomorph, *d*(+)α-hydroxyglutaric acid, which is formed in this reaction, and it has been shown that the hydroxyglutaric dehydrogenase of animal tissues is only capable of activating the *l*-form. Mayer [1931] discusses the formation of methylglyoxalylacetic acid from fructose via laevulic acid. Some experiments which have been carried out to test this hypothesis showed that neither laevulic acid nor methylglyoxalylacetic acid is metabolized by animal tissues.

Recent work [Weil-Malherbe, 1936; 1937, 1; Krebs & Johnson, 1937] has made it clear that ketoglutaric acid is a very important intermediate in the later stages of substrate oxidation. Since it can be reduced in a reversible reaction, it has to be added to the number of potential hydrogen acceptors by which an anaerobic metabolism is, to a certain extent, made possible. The reversibility of the hydroxyglutaric dehydrogenase shows that the reduction can be catalysed by this enzyme. It is still uncertain if there is another mechanism operating, e.g. a mutase action, by which the decarboxylation of α-ketoglutaric acid may be coupled with its reduction.

Activity of the enzyme. It is difficult to compare the activity of hydroxyglutaric dehydrogenase with that of other dehydrogenases as long as the mechanism of its maximum activation is not known. An entirely wrong impression would have been obtained if, for example, methylene blue had been used instead of pyocyanine. We do not know, however, whether the activation by pyocyanine really represents the optimum or whether it could not be further increased by the use of its natural carrier, as yet unknown. Assuming that with pyocyanine the optimum activation is approximately reached it must be admitted that the activity of the enzyme is smaller than that of other dehydrogenases. If the same extract from pig's heart is used as preparation of the dehydrogenases it appears that the initial rate of reaction of the hydroxyglutaric dehydrogenase is about 10 times smaller than that of succinic dehydrogenase and about 3 times smaller than that of lactic or malic dehydrogenase, while it is about equal to that of β-hydroxybutyric dehydrogenase.

Preparations and acknowledgements

l(−)α-Hydroxyglutaric acid was prepared according to Fischer & Moreschi [1912]. $[\alpha]_D$ of the Na salt −8·66° (Fischer & Moreschi 8·65). *d*(+)α-Hydroxyglutaric acid was made from α-chloroglutaric acid by the method of Fischer & Moreschi, whereas for the preparation of α-chloroglutaric acid the method of Jochem [1900] was found preferable. α-Ketoglutaric acid was prepared according to Neuberg & Ringer [1915]. Since the recrystallization presented difficulties, the acid was converted into the calcium salt, acidified, extracted with ether and the ether removed *in vacuo*. M.P. 113°.

Cytochrome *c* was prepared from baker's yeast [Keilin, 1933], as was coenzyme I [Myrbäck & Oertenblad, 1935]. ACo of the coenzyme preparation approx. 100,000. Adenosinetriphosphate was made by the method of Lohmann [1931]. Yellow enzyme and coenzyme II (purity 1) were

given to Dr Dickens by Prof. Warburg. The crude mixture of coenzymes from horse blood was prepared by Dr Dickens according to Warburg *et al.* [1935]. Phenazine derivatives (with the exception of pyocyanine which was a Hoffmann-La Roche product) were prepared by Dr H. McIlwain, Armstrong College. Lactoflavin was presented by Bayer Products, Ltd.

SUMMARY

l(−)α-Hydroxyglutaric acid is oxidized to α-ketoglutaric acid by a dehydrogenase which is found in many animal tissues. The properties of this enzyme have been studied in detail. Its action does not depend upon any coenzyme. A carrier is necessary for the reaction with molecular oxygen; pyocyanine and a few closely related phenazine derivatives were found most active, whereas cytochrome C is inactive. H_2O_2 is formed during the reaction with oxygen and can be demonstrated in presence of cyanide.

The enzyme action is reversible. The potential of the system lies probably in the neighbourhood of −0·07, though a direct measurement was not possible.

The optical isomeride, *d*(+)α-hydroxyglutaric acid, is not attacked by the dehydrogenase.

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